RESEARCH PLAN PROPOSAL

ISOLATION AND CHARACTERIZATION OF CHLORPYRIFOS TOLERATING MICROORGANISMS AND THEIR PLANT GROWTH PROMOTING EFFICACY

For registration to the degree of

Doctor of Philosophy

IN THE FACULTY OF SCIENCE

THE IIS UNIVERSITY, JAIPUR

Submitted by

Disha Pamecha

ICG/2017/24539

Under the Supervision of

Dr. Radhika Sharma

Associate Professor

Department of Biotechnology

The IIS University, Jaipur

ISOLATION AND CHARACTERIZATION OF CHLORPYRIFOS TOLERATING MICROORGANISMS AND THEIR PLANT GROWTH PROMOTING EFFICACY

INTRODUCTION

A fast growing population of 1.27 billion and agriculture being the primary source of livelihood for about 58 per cent presents a critical need for intensification in agriculture for India's development. Indian agricultural sector is likely to grow at 2.1 per cent in 2018 followed by industrial sector, according to the Economic Survey 2017-18.

While India has achieved grain self-sufficiency but the production is, resource intensive, cereal centric and regionally biased. Owing to the above mentioned factors immense crop protection assumes an important place in the agricultural economy (Devi *et al*., 2017).

The Indian pesticide industry has witnessed a massive boost in terms of pesticide production. Consequently, the consumption of pesticides in modern agriculture practices has become an important tool of crop production in many regions. Pesticides are significantly used in areas of crop production to minimize infestations by pests and protect yield losses and reduction of product quality. With the primary concern to minimize deleterious effects caused by viruses, bacteria, fungi and insects extensive use of such chemicals have been observed often at very high levels with unscientific patterns of application. Large complex of insect pests ranging from borers to root feeding insects are responsible for heavy losses of commercial crops, both quantitatively and qualitatively. In order to reap maximum yields, the farmers resort to pesticide application to combat the pest problem (Anuja, 2005). Further, pesticides play a significant role in keeping plants away from many dreadful diseases and thereby reducing crop loss. However, exposure to pesticides both environmentally and occupationally is the root cause of a wide range of human health problems (Singh and Abhilash, 2009).

There is overwhelming evidence that some of these chemicals do pose potential risks to the ecosystem in general and human beings in particular (Ashiq *et al*., 2016), (Devi *et al*., 2017). The applied pesticides reach the target pests by only 1% (Raju *et al*., 2016) and the residues stay in in the environment (air, water and soil) for variable periods of time causing contamination of soil and water bodies. This periodic employment and repetition in the long

run leads to their accumulation in the natural resources such as water and soil, endangering the entire population by their multifaceted toxicity (Ashiq *et al*., 2016). Amongst the most important problems associated with pesticide application are their possible persistence in the environment and therefore, their possible incorporation into the food chain affects ecosystem and human beings (Liu and Xiong, 2001).

Pesticide exposure inflicts chronic and acute threats to human health. Studies have increasingly linked repeated pesticide exposure to immune suppression, hormone disruption, diminished intelligence, reproductive abnormalities and cancer (Singh and Abhilash, 2009).

Organophosphorus pesticides (OPPs) are widely used pesticides or chemical warfare agents, due to their high toxicity towards insects, mammals and other animals their extensive use contaminate the environment. The risk of its percolation in soil increases when pesticide is not degraded and biological persistence poses health risk to humans. Chlorpyrifos is an organophosphorus insecticide widely used across the world. It is used for controlling pests during the production of agricultural commodities and for treating the soil in which different crops are grown. It is a broad spectrum pesticide with trade names Dursban, Lorsban and Spannit, displaying insecticidal activity against a wide range of insect and arthropod pests (Bhagobaty *et al*., 2007).

Chlorpyrifos-oxon and 3, 5, 6-trichloro-2-pyridinol (TCP), the two potent degradation products of chlorpyrifos, have been found in groundwater and are very much stable. In soil, chlorpyrifos may remain biologically active for periods ranging from days to months. Dosage rates, soil type, soil moisture and organic matter content, temperature and insecticide formulation are among the factors which influence biological persistence (Read, 1976; Tashiro and Kuhr, 1978). Many methods for the detection of chlorpyrifos residues in the environment, including in water, soil and some fruits and vegetables, have been reported, but most of these methods are based on multi residue analysis, and most studies investigated chlorpyrifos and other pesticides but did not focus on the metabolites of these pesticides, such as the toxic metabolite of chlorpyrifos 3, 5, 6-trichloro-2-pyridinol-TCP (Yucel *et al.,* 1999).

Many chemical and enzymatic methods for remediation of pesticide contaminated soils have been employed. But, none of them gave satisfactory results. Microorganisms being the cheapest and easily available sources are most readily used for remediation of contaminated soils. Bioremediation which includes the gainful utilization of microorganism for the metabolism or biodegradation of target pollutants into safer and innocuous products is

amongst the potent technologies that are being used globally for the restoration of contaminated sites.

Insecticides and their degradation products, generally get accumulated in the top soil and influence not only the population of various groups of soil microbes but also their biochemical activities like nitrification, ammonification, decomposition of organic matter and nitrogen fixation (Agnihotri *et al*., 1981). Microorganisms play an important role in degrading synthetic chemicals in soil (Alexander, 1981).

REVIEW OF LITERATURE

Pesticide is a general term for substances, which are used to poison pests (weeds, insects, rodents etc.). Synthetic pesticides have been popular with farmers, because of their widespread availability, simplicity in application, efficacy and economic returns. But they also have huge environmental costs.

Pesticide consumption in India-

After India's Green Revolution started, the consumption of pesticides in India has increased several hundred folds, from 154 MT in 1954 to 88,000 MT in 2000-2001. According to industry estimates, the pesticide use has high growth potential in India, as the use of agricultural pesticides is markedly low at 0.54 kg /ha as against 3.7 kg/ha in USA and 2.7 kg/ha in Europe. Notwithstanding the fact that overall consumption of pesticides in India as a whole is low than that used in the developed countries of the world, there is still a widespread contamination of water, soil and air with pesticide residues. In India, among different states maximum consumption of pesticides-1999-2000 was in Uttar Pradesh (7459 MT) followed by Punjab (6972 MT), Haryana (5025 MT), Andhra Pradesh (4054 MT), Gujarat (3646 MT). Leading pesticides used in India include monocrotophos (10700 MT- highest consumed), acephate (6400MT), endosulfan (5600 MT) and chlorpyrifos (5000 MT - fourth highest consumed) (Pesticide Information, Volume XXVIII, No. 3, October- December 2002).

The production of pesticides started in India in 1952 with the establishment of a plant for the production of BHC near Calcutta, and currently, India is the largest producer of pesticides in Asia and ranks twelfth in the world for the use of pesticides (Singh and Abhilash, 2009).

Chlorpyrifos industry has developed fast in the past years worldwide, especially in some developing countries, such as China and India. The demand for chlorpyrifos has witnessed rapid growth in many countries in recent years, which has promoted its large capacity expansion and technology innovation, resulting in supply surplus as well as production cost decrease (CCM press release, China, 2011).

Chlorpyrifos:

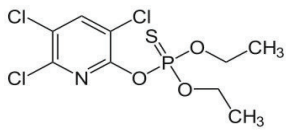
Chlorpyrifos is one of the universally used organophosphorus pesticides in crop production. The estimated consumption of technical grade chlorpyrifos in India during 2002-03 was 5,000 MT (Jisha *et al*., 2018). It is used both for agricultural pest control and in households

as a termiticide. Commercially, it is available in various brand names like Dursban, Agromil, Dhanwan, Dorson, Dhanwan, Lorsban and Omexan to name a few (Bhagobaty *et al*., 2007).

Chlorpyrifos (O, O-diethyl O-3, 5, 6-trichloro-2-pyridyl phosphorothioate), esters derived from phosphoric acid, is one of the broad spectrum organophosphate pesticide used against sucking, chewing and boring insects (Hamsavathani *et al.,* 2017). It can easily enter the human food chain and has more victims to its credit than carcinogenic air pollutants like polycyclic aromatic hydrocarbons (PAHS). Chlorpyrifos causes hazardous effects to the environment and also toxic to human beings resulting in headache, nausea, muscle twitching, convulsions, birth defects and even death (Hamsavathani *et al*., 2017).

Chlorpyrifos, being the second largest selling organophosphorous chemical in India, interferes with the normal functioning of the central nervous system, including the brain, by inhibiting acetylcholinesterase, an enzyme that modulates the amount and levels of the neurotransmitter acetylcholine and thus disruptin the nerve impulse (Hamsavathani *et al*., 2017). Symptoms of acute poisoning include headache, nausea, muscle twitching and convulsions and in some extreme cases even death. Human birth defects have also been associated with exposure to chlorpyrifos and its products. Chlorpyrifos also affects the male reproductive system. It is toxic to a variety of beneficial arthropods including bees, ladybird beetles and parasitic wasps (Bhagobaty *et al*., 2007).

Chlorpyrifos is available in a variety of formulations, such as granules, wettable powder, dustable powder and emulsifiable concentrate. It has a very low solubility in water (2 mg/L) but is readily soluble in most organic solvents and is used for the control of major insects and pests affecting a wide range of crops of cereals, cotton and vegetables etc. (Jisha *et al*., 2018). Dursban is the most toxic organophosphorus compound and is more toxic than organochlorine compounds (Veeraiah *et al.,* 2001).



Structure of chlorpyrifos

The physical and chemical properties of a compound determine its behaviour in the environment. Although environmental variables are important in modulating transformation

and transport processes, the basic pattern of persistence and partitioning is fundamentally derived from the chemical character of the compound.

The following are the characteristics of chlorpyriphos: (Source: George, 2005)

Common name: Chlorpyriphos
IUPAC name: O, O-diethyl O-3, 5, 6-trichloro-2-pyridyl phosphorothioate
Empirical formula: $C_9H_{11}C_{l3}NO_3PS$
Molecular weight: 350.6
Colour: White/colourless crystalline solid (pure active constituent)
White to light yellowish-brown crystalline solid (technical grade)
Odour: Odourless
Melting point: 41 to 43.5°C
Vapour pressure: 1.87 x 10-5 mm Hg at 25°C
Solubility in water: 1.2 mg/L at 25°C
Solvent solubility (g/100 g) - Acetone: 650
Chloroform: 630
Benzene: 790
Diethyl ether: 510
Stability: Stable in air (non-volatile) and is not sensitive to UV radiation. It is stable to neutral and weakly acidic solutions, but is hydrolysed by strong bases.

Toxicity and mode of action

Chlorpyrifos works as a synaptic poison displaying a wide spectrum of insecticidal activities used to check insects attacking corn, cotton, citrus fruits, fruits, potato, beets and beans. According to the WHO classification, chlorpyrifos belongs to class II pesticides with moderate toxicity. It is moderately persistent in nature and even after 2 months its residual effects can be detected in soil. Substantial residues of chlorpyrifos were found in tomatoes, cotton seed, and in oil of groundnut, safflower, and mustard. Chlorpyrifos functions in three ways:

- By contact
- ingestion,
- vapour action

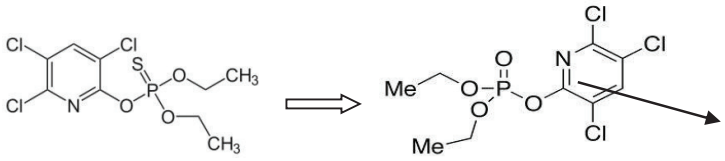
It has been reported that the absorption of chlorpyrifos is 70% through oral route while less than 3% of skin. The organophosphates readily bio-transform in the liver and do not accumulate in the human organs. Exposure to chlorpyrifos is responsible for acetylcholine accumulation in skeletal muscles and brain which interferes with the transmission of impulse at nerve endings (Gilani *et al*., 2015).
The chlorpyrifos toxicity can be broadly classified into two groups:

Chronic toxicity: Repeated or prolonged exposure to organophosphates may result in the same effects as acute exposure including the delayed symptoms. Other effects reported in workers repeatedly exposed include impaired memory and concentration, disorientation, severe depressions, irritability, confusion, headache, speech difficulties, delayed reaction times, nightmares, sleepwalking, and drowsiness or insomnia. An influenza-like condition with headache, nausea, weakness, loss of appetite, and malaise has also been reported (US PHS, 1989). When technical chlorpyrifos was fed to dogs for 2 years, increased liver weight occurred at 3.0 mg/kg/day. Signs of cholinesterase inhibition occurred at 1 mg/kg/day. Rats and mice given technical chlorpyrifos in the diet for 104 weeks showed no adverse effects other than cholinesterase inhibition (US EPA, 1989).

Acute toxicity: Chlorpyrifos is moderately toxic to humans. (US EPA, 1989) Poisoning from chlorpyrifos may affect the central nervous system, the cardiovascular system, and the respiratory system. It is also a skin and eye irritant. While some organophosphates are readily absorbed through the skin, studies in humans suggest that skin absorption of chlorpyrifos is limited (Gallo and Lawryk, 1991). Symptoms of acute exposure to organophosphate or cholinesterase-inhibiting compounds may include the following: numbness, tingling sensations, ill-coordination, headache, dizziness, tremor, nausea, abdominal cramps, sweating, blurred vision, difficulty breathing or respiratory depression, and slow heartbeat. Very high doses may result in unconsciousness, incontinence, and convulsions or fatality (Gallo, 1991).

Chlorpyriphos applied topically to fire ants (*Solenopsis richteri*) is rapidly transported from the external cuticular surface to the hemolymph and organs, and greater than 90 per cent of the applied dose has been reported to get absorbed within 4 hrs (Chambers *et al*., 1983).

Chlorpyrifos is directly toxic to the nervous system. In addition, it is transformed inside animals to chlorpyrifos-oxon which is about 3000 times more potent against the nervous system as compared to chlorpyrifos itself (Goodman *et al.*, 1985).



AChE inhibition

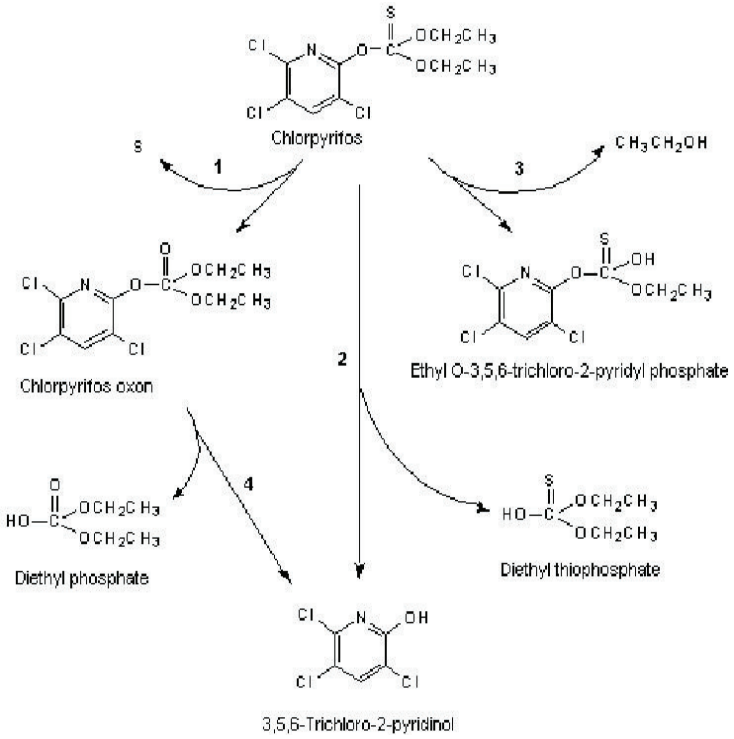
Chlorpyrifos chlorpyrifos-oxon

The toxicity of insecticidal organophosphorus and carbamate esters to animals is attributed to their ability to inhibit acetylcholinesterase (AChE, choline hydrolase, EC 3.1.1.7), which is a class of enzymes that catalyzes the hydrolysis of the neuro-transmitting agent acetylcholine (ACh). The inhibition of AChE and related esterases, often referred to as serine hydrolases, has been clearly demonstrated to be the result of an actual chemical reaction between the enzyme and the organophosphate or carbamate ester. The phosphorylated or carbamylated enzyme is no longer capable of affecting the hydrolysis of ACh; this results in a build-up of the neurotransmitter at a nerve synapse or neuromuscular junction (Fukuto, 1990).

Organophosphorus pesticides acts by inhibiting acetylcholinesterase by phosphorylation and elicit a spectrum of cholinergic effects. The primary mechanism of toxicity for organophosphorus pesticides, like chlorpyrifos, is cholinesterase (ChE) inhibition. The inhibition of the enzyme acetylcholinesterase (AChE) results in the buildup of acetylcholine (ACh) at choline receptors, causing continual nerve stimulation (Giesy *et al.,* 1999).

Kanekar *et al*. (2004) has also reported that on exposure to organophosphates acetylcholinesterase enzyme is not able to function, causing accumulation of acetylcholine, which interfere with the transmission of nerve impulses at the nerve endings.

Chlorpyrifos is a relatively weak AChE inhibitor compared to its metabolite chlorpyrifos oxon (El- Merhibi *et al*., 2004), thus toxicity is initiated by the formation of chlorpyrifos oxon by oxidative desulfuration (Giesy *et al*., 1999). Factors influencing the toxicity of chlorpyrifos between species and groups include metabolic rate, the number of target sites available for chlorpyrifos metabolism to chlorpyrifos oxon (Chambers and Carr, 1995) organism surface area, and lifestage (El-Merhibi *et al*., 2004).



Metabolic pathway of chlorpyrifos (Dhanya, 2014)

Bioremediation of Chlorpyrifos

Adverse effects of chlorpyrifos on all the life forms, directly or indirectly, put forward a need to eliminate it from the contaminated soils and waters. Degradation of pesticides occurs both by biotic and abiotic pathways, however microbial degradation is primarily responsible for breakdown and detoxification of pesticides in soil. Actions of microorganisms convert the toxic parent compounds to intermediate and less toxic metabolites. Degradation of chlorpyrifos using microorganisms has proven to be a cheap, efficient and economical method in improving the quality of environment (Gilani *et al*., 2015).

Bioremediation which includes the gainful utilization of microorganism for the metabolism or biodegradation of target pollutants into safer and innocuous products is amongst the potent technologies that are being used globally for the restoration of contaminated sites (Anuja, 2005).

Bioremediation is the process which utilizes naturally occurring microorganisms to breakdown hazardous substances unto less toxic substances. It uses relatively low-cost, low

technology techniques, which generally have a high public acceptance and can be carried out on site (EPA).

Due to the ill effects of chlorpyrifos on environment and growing toxicity, the need was generated to degrade the residues of chlorpyrifos to less harmful products. Many researches were done to find the efficient ways of degradation including chemical degradation.

Chemical degradation of pesticides have been reported by several researchers with 3, 5, 6-trichloro-2-pyridinol (TCP) as the intermediate compound. Chemical degradation has its own limitations as it does not completely degrades the TCP which is formed as an intermediate, it persists in soil even after degradation and it is a stable product. Thus, we need to find out ways where we can achieve complete degradation of chlorpyrifos.

Biodegradation of Chlorpyrifos by Bacterial Communities

The bacterial communities are capable of degrading pollutants more efficiently as they have the wonderful asset of catabolic genes to survive in various ecological niches over a fairly extensive range of pH, temperature, O2 and heavy metal concentrations. Their potential can be utilized for the biodegradation of chlorpyrifos (Yadav *et al.*, 2015).

Unlike in the case of other organophosphates, however, there have been no reports of enhanced microbial degradation of chlorpyrifos since its first use in 1965 until, recently when in a study by Feng and his co-workers in 1997, a pure culture of bacteria capable of using 3, 5, 6-trichloro-2-pyridinol (TCP) as the sole source of carbon and energy under aerobic conditions was isolated. Mallick *et al*., (1999) reported the rapid degradation of chlorpyrifos, added to a mineral salt medium as a sole carbon source by *Flavobacterium* sp. and *Arthrobacter* sp.

Huang *et al*., (2000) reported the degradation of chlorpyrifos by aerobic microbial processes in poultry and cow-derived effluents. Further, the microbes involved in the degradation process were analysed by denaturing gradient gel electrophoresis (DGGE) of PCR amplified 16sRNA genes and it was observed that a single band became dominant in effluents during chlorpyrifos degradation, which indicated that a single aerobic-bacterial population was capable of degrading chlorpyrifos.

43 cultures of bacteria from various soil samples were collected from different regions of Punjab and screened for the degradation of chlorpyrifos by Kahlon and Saurabh (2003). Four

isolates were found to grow efficiently which were further selected for the bioaugmentation of pesticide degradation in the contaminated soil.

Singh *et al*., (2004) worked on biodegradation of chlorpyrifos by *Enterobacter* strain B-14 and its use in bioremediation. It was found that the addition of strain B-14 (106 cells/g) to soil inoculated with chlorpyrifos degrading bacteria, treated with 35 mg of chlorpyrifos/kg resulted in a higher degradation rate.

Two recently isolated CP-degrading bacteria, *Stenotrophomonas* sp. and *Sphingomonas* sp., could utilize CP as the sole source of carbon and phosphorus, but they did not degrade TCP (Li *et al*., 2007; Yang *et al*., 2006). Mineralization of TCP has been reported by Pseudomonas sp. (Feng *et al*., 1997), and by a strain of Alcaligenes sp. capable of biodegrading both chlorpyrifos and TCP (Yang *et al*., 2005).

Chlorpyrifos has been reported to be degraded co-metabolically in liquid media by *Flavobacterium* sp. and *Pseudomonas diminutive*, which were initially isolated from a diazinon treated field and by parathion enrichment, respectively (Singh and Walker, 2006).

Agus Sabdono (2007) found that a bacterium which utilizes chlorpyrifos as a sole source of carbon and energy isolated from coral surface utilized chlorpyrifos up to 25 mg l^{-1}. This strain demonstrated greatest similarity to members of the order *Bacillales* and was closest to members of the *Bacillus firmus* group.

Pseudomonas aeruginosa, common gram negative bacterium found in soil, has been found to have potential to degrade chlorpyrifos (Fulekar & Geetha, 2008).

Five microbial consortia from chlorpyriphos contaminated sites by selective enrichment techniques were isolated by Khanna *et al.,* 2009. Out of them two were the promising consortia with degradation efficiency of 72 and 70 per cent respectively.

Zhiyuan Liu *et al*., 2012 worked on the bacterial degradation of chlorpyrifos in liquid medium and found that it degraded under the condition of 30˚C, pH 7.0, concentration of chlorpyrifos below 150 mg L^{-1}, with a degradation rate up to 78.85%.

Briceno *et al*., 2013 studied about the enhanced removal of chlorpyrifos and pentachlorophenol by single cultures and consortia of free and immobilized *Streptomyces* strains. The antagonism between the strains and their tolerance to the pesticides was evaluated. Results obtained showed high tolerance levels against these pesticides and no antagonistic effects were seen. *Streptomyces* sp. M7 showed high degradation of chlorpyrifos (99.2%) and pure culture of *Streptomyces* sp. A5 showed degradation rate of 10.6% for pentachlorophenol.

Paenibacillus polymyxa and *Azospirillum* were reported to degrade organophosphorous pesticides-Chlorpyrifos, Chlorpyrifos-methyl and cyanophos in mineral salt medium. The pesticides were used as sole carbon and phosphorus source and it was also found that *Paenibacillus* was more effective than *Azospirillum* in degrading these pesticides (Romeh *et al*., 2014).

Chlorpyrifos degradation is of high importance as it shows high mammalian toxicity. *Pseudomonas aeruginosa DKC2* showed good growth in basal salt medium suggesting their ability to use chlorpyrifos as sole source for carbon and energy (Bhatia *et al.,* 2013).

Acinetobacter calcoaceticus was isolated from rhizosphere contaminated with chlorpyrifos. The bacteria was capable of utilizing chlorpyrifos as the sole source of carbon for growth and it also showed multiple plant growth promoting traits (Lei *et al.,* 2014).

The bacterial isolate *Paracoccus* sp. TRP isolated from activated sludge sample, degrades 100% of CP in 4 days while Serratia sp. is able to degrade the same in 18 h only which indicates that Serratia sp. is more efficient than Paracoccus sp. (Yadav *et al*., 2015).

yFp tagged *Mesorhizobium sp. HN3* showed enhanced plant growth and degradation of chlorpyrifos and its metabolite 3, 5, 6 trichloro-2-pyridinol (TCP). Significantly lower residues of the pesticides were observed in plants grown in inoculated soil suggesting its involvement in chlorpyrifos degradation (Jabeen *et al.,* 2015).

Akbar *et al.*, 2016 isolated two bacterial strains JCp4 and FCp1 having chlorpyrifos degradation potentials of 84.4% and 78.8% respectively. These strains were identified as *Achromobacter xylosoxidans* (JCp4) and *Ochrobactrum sp.* (FCp1) based on 16s rRNA sequence analysis.

To date, several chlorpyrifos-degrading bacterial strains were isolated *including Enterobacter sp, Pseudomonas putida, Ps. aeruginosa*, *Ps. desmolyticum*, *Ps. resinovorans*, *Ps. fluorescens* , *Bacillus subtilis*, *Bacillus sp and Micrococcus sp*, *B. firmis*, *B. cereus*, *Stenotrophomonas* , *Serratia marcescens, Klebsiella oxytoca, Achromobacter sp, Ochrobactrum sp*, *Alcaligenes faecalis, Mesorhizobium*, *cellulomonas fimi*, *Gordonia*, *B. polymyxa*, *Kocuria sp*, *Staphylococcus sp, Streptococcus sp, azomonas sp, Flavobacterium sp* were extensively studied in degradation of chlorpyrifos (Hamsavathani *et al.*, 2017).

Biodegradation of Chlorpyrifos by Fungal Communities

Fungi are critical to the biogeochemical cycle and are responsible for the degradation of various xenobiotic compounds in the biosphere. Mycelial growth and extracellular enzymes provide them an edge over other microorganisms like yeasts and bacteria (Carranza *et al*., 2016).

Several species of *Aspergillus*, *Trichoderma harzianum* and *Penicillium brevicompactum* have been reported to utilize chlorpyrifos as sources of phosphorus and sulphur (Yadav *et al*., 2005).

The degradation of CP has also been reported by other workers using fungal communities, such as *Trichoderma viridae*, *Aspergillus niger*, *Acremonium* sp. (GFRC-1) and *Cladosporium cladosporioides* (Yadav *et al*., 2005).

Maya et al. (2012) studied biodegradation of CP by fungal species such as *Aspergillus*, *Penicillium* and *Eurotium* isolated from agricultural soil (Carranza *et al*., 2016).

Factors affecting rate of degradation of chlorpyrifos

Different factors influencing the rate of bioremediation are temperature, pH, nutrient source of carbon and nitrogen. Degradation of chlorpyrifos by soil microorganisms is strongly affected by soil pH.

Pseudomonas exhibits maximum degradation at pH 8 because at high pH, enzymes have optimum activity and able to degrade chlorpyrifos. In soils with high pH, TCP is metabolized into CO_2, H_2O, and NH_4^+ through reductive dechlorination pathway by a Pseudomonas. Soil organic matter may enhance microbial activity by co-metabolism and decrease pesticide biodegradation by motivating pesticide adsorption process. Temperature plays an important role in the degradation of pesticide by depending on the molecular structure of the pesticide. Highest growth and activity of microorganisms occur at 25–35 °C in the soil and the rate of

degradation is optimum at temperature ranges from 25 to 40 °C in mesophilic conditions. Moisture plays vital role in the transformation process and it is directly proportional to the increase of water content in soil. Chlorpyrifos can persist for several months in environment of limited microbial activity, light and water (Gilani *et al.*, 2015), (Tang *et al.*, 2017).
Cladosporium cladosporioides Hu-01 was able to transform CP provided at concentration 50 mg/l. The complete mineralization of TCP was achieved after 6 days of incubation which was confirmed by gas chromatography-mass spectroscopy analysis (Raju and Supreeth, 2017)

Plant growth promoting activity by pesticide degrading bacteria

The inoculation of plants with beneficial bacteria to increase plant growth and protect plants from diseases is a promising strategy in modern crop production systems. Among bacterial inoculants are plant growth promoting rhizobacteria (PGPR), which are free living rhizosphere bacteria that promote plant growth and development by several mechanisms (Myresiotis *et al.*, 2014).

Direct plant growth promotion by PGPR involves the stimulation of root growth by producing plant hormones or facilitating the uptake of certain nutrients from the soil by various mechanisms such as nitrogen fixation, phosphate solubilization and synthesis of siderophores. They also include the production of plant hormones like auxins, gibberlins and cytokinins. PGPR can also indirectly induce plant growth by protecting plants against pathogens, a process known as biocontrol activity (Myresiotis *et al.*, 2014).

The inoculation of crops with PGPR may constitute an alternative way to increase productivity in a sustainable manner, reducing the use of agrochemicals which often pollute the environment. A number of studies have shown that PGPR are potential growth enhancers in several crops. In particular, successful examples of inoculation of corn, wheat, tomato and other crops with PGPR strains of the genera *Azospirillum, Bacillus, Pseudomonas* and *Enterobacter* have been achieved in laboratory and field experiments (Myresiotis *et al.*, 2014).

Strains of *Gordonia, Klebsiella, Paenibacillus, Serratia* are among others. *Bacillus* and *Pseudomonas* spp. have a wide distribution and are most extensively studied. In recent years, several PGPR-based products became commercially available in many countries, and many more are currently under development (Choudhary and Johri 2009).

Among the bacilli, strains of *Bacillus subtilis* are the most widely used PGPR because they are able to produce a broad spectrum of antibiotics, and effectively colonize the rhizosphere and control soil borne pathogens (Cazorla *et al.,* 2007).

Pseudomonas, Azospirillum, Agrobacterium, Bacillus, Enterobacter and *Flavobacterium* are some of the genera which include PGPR strains able to degrade organic and inorganic contaminants in soil (Zhuang *et al.,* 2007). In addition to biopesticides, many soil applied synthetic pesticides are used against weeds, pests and pathogens during conventional or integrated crop production programs. Furthermore, the attention has been paid on bioremediation of contaminated soils with PGPR (Jiang *et al.,* 2008).

Chlorpyrifos degradation is of high importance as it shows high mammalian toxicity. *Pseudomonas aeruginosa DKC2* showed good growth in basal salt medium suggesting their ability to use chlorpyrifos as sole source for carbon and energy. It also showed various plant promoting activities like phosphate solubilization, protease activity and Indole-3-acetic acid production (Bhatia *et al.,* 2013).

Acinetobacter calcoaceticus was isolated from rhizosphere contaminated with chlorpyrifos. The bacteria was capable of utilizing chlorpyrifos as the sole source of carbon for growth and it also showed multiple plant growth promoting traits of phosphate solubilization, Indole-3-acetic acid and siderophore production (Lei *et al.,* 2014).

yFp tagged *Mesorhizobium sp. HN3* was shown to efficiently colonize the rhizosphere of ryegrass planted in chlorpyrifos contaminated soil. It showed enhanced plant growth and degradation of chlorpyrifos and its metabolite 3, 5, 6 trichloro-2-pyridinol (TCP). Significantly lower residues of the pesticides were observed in plants grown in inoculated soil which might be attributed to the efficient root colonization of HN3yFp, suggesting its involvement in chlorpyrifos degradation inside the roots and rhizosphere of plants (Jabeen *et al.,* 2015).

A recent study was done by Pratibha Yadav *et al*. (2015), on remediation of organophosphate pesticide Methyl parathion using 4 Rhizobacteria isolates- RB1, RB2, RB3 and RB4. These isolates were grown on a medium containing methyl parathion as sole carbon and/or nitrogen source. RB1 and RB4 were found to have maximum enzyme activity for degradation of methyl parathion. Plant bioassays were also performed where on inoculation with these

isolates the plant growth was observed in terms of leaf count, seed vigour, and growth increment in radical length and percentage increment in seed germination.

Shamsa Akbar *et al.* (2016) isolated two bacterial strains JCp4 and FCp1 having chlorpyrifos degradation potentials of 84.4% and 78.8% respectively. These strains were identified as *Achromobacter xylosoxidans* (JCp4) and *Ochrobactrum sp.* (FCp1) based on 16s rRNA sequence analysis. These strains also showed plant growth promoting activities like indole acetic acid production and phosphate solubilization in both absence as well as presence of chlorpyrifos. In plant growth experiments conducted on *Vigna unguiculata*, it was found that these strains enhance plant growth significantly in terms of plant weight and plant length and it was noted that strains shows increased degradation rate of chlorpyrifos in planted soil.

OBJECTIVES

The objectives of the study are:

1. To isolate the microorganisms capable of degrading chlorpyrifos present in the soil.
2. To study the chlorpyrifos degradation b isolated microorganisms and determination of their maximum tolerance to chlorpyrifos.
3. Morphological, molecular and biochemical characterization of the isolated microorganism.
4. Response Surface Modification Optimization for pesticide degradation.
5. Study of the plant growth promoting efficacy of the characterized microorganism and its interaction with *Pennisetum glaucum* (Pearl millet).

PPROPOSED METHODOLOGY

(WORK PLAN)

I. Chemicals and Media

- The technical grade Chlorpyrifos will be purchased from a pesticide manufacturer.
- Luria Bertini Broth (pH 7.2)
- Luria Bertini Agar (pH 7.0)
- Czapek Dox Agar (pH 7.3)
- Minimal Salt Media for bacteria (pH 7)
- Minimal Salt Media for fungus

II. Sample Collection and Preparation

- Soil samples from the top layer (10-15 cm) will be collected using a soil auger from four different areas of those agricultural fields in Durgapura Research Station of Rajasthan Agricultural University where chlorpyrifos is being used on vegetation. Samples will be pooled and collected in labelled sterilized polythene bags to avoid any external contamination. The soil sample containing polythene bags will be transported to the laboratory and stored at 4°C to maintain its biological activity (Kumar *et al*., 2017).
- Soil samples will be sieved through a mesh of pore size =<2mm to remove any stones and debris (Hua *et al*., 2008). Soil moisture will be maintained at 40% by regular spraying of distilled/deionized water for the development of aerobic microbial consortia utilizing chlorpyrifos as sole carbon and energy source (Gong *et al*., 2016).
- The physico-chemical characteristics of the soil affect the degradation of pesticides thus rendering their study an important task. 'The following physico-chemical characteristics of the collected soil will be determined:

PHYSICAL CHARACTERISTICS	CHEMICAL CHARACTERISTICS
Colour	pH
Odour	Total nitrogen
Sand	Heavy metals
Silt	Na^{+}
Clay	Mg^{+2} / SO_4^{-2}
Organic matter	Ca^{+2}
Water holding capacity	Cl^{-}

III. Isolation and purification of Chlorpyrifos degrading microbes by media enrichment

- 10 g soil sample will be taken and added to Mineral Salt Media (MSM) supplemented with 20 ppm of chlorpyrifos. It will be incubated at 37℃ in a shaking incubator at 120 rpm for 5 days. The carbon source in MSM media will be replaced by chlorpyrifos (Jisha *et al.*, 2018).
- After 5 days of incubation, soil samples will be serially diluted from 10^{-2} - 10^{-6} and 1 ml from each will be plated on Nutrient Agar and Czapek Dox Agar enriched with 20 ppm of chlorpyrifos, incubated at 30℃ for 3 and 5 days for obtaining bacterial and fungal strains respectively.
- Individual distinct colonies of strains utilizing chlorpyrifos as sole carbon source on chlorpyrifos enriched agar will be selected; sub cultured and maintained on Nutrient Agar and Mineral Salt Agar slants through streaking (Jisha *et al*, 2018).

IV. Preparation of microbial inoculum for chlorpyrifos degradation

- Aliquots of 50 ml MSM will be prepared by inoculating isolates using sterilized loops, incubated at 37℃ and 30℃ for bacterial and fungal strains respectively for 24 -48 hours at 150 rpm (Ishag *et al*, 2016).
- For preparing an inoculum, desired amount of culture will be pelleted by centrifugation at 8000 rpm for 7 minutes. The supernatant will be discarded and pellet will be washed thrice with 25 ml of 0.0125 M phosphate buffer (pH 7.2) and quantified by dilution plate count technique.
- For all experiments, 10^6 cells ml^{-1} will be used (Singh *et al*, 2004).

V. Screening of chlorpyrifos degradation

The aim of this experiment will be to evaluate the capability of the isolated and identified microbial strains to degrade chlorpyrifos.

- 10 ml of MSM from stock flask will be taken in a sterilized test tube. 1 ml of inocula will be added to it.
- The cultured test tube will be incubated at 37℃ and 30℃ for bacterial strains and fungal strains respectively with 20 ppm chlorpyrifos for 72 hours.
- 10 ml from 20 ppm will be added to another set of MSM supplemented with chlorpyrifos to obtain 40 ppm concentration. This will be continued with increasing concentration of chlorpyrifos i.e. 60, 80, 100 ppm.
- Control sets without inoculum will be incubated under same conditions.
- Post incubation for 72 hours, treated cultures will be analysed for chlorpyrifos degradation.

a) UV-Vis Spectrophotometry
    - Treated cultures will be centrifuged at 800 rpm for 10 min to separate the microorganisms from the media. The supernatant will be removed by careful decanting and placed in a clean and sterilized test tube.
    - The pesticide degradation in the media will be analysed at a regular interval by taking optical density of the cell free extract of the minimal media emended with chlorpyrifos at the absorbance maxima of chlorpyrifos i.e. 300nm (Sharma *et al*, 2016).

b) High Performance Liquid Chromatography
    - The estimation of degraded chlorpyrifos in microbial strains will be carried out using High Performance Liquid Chromatography (HPLC).
    - For determining the degradation potential, cultures grown in MSM will be taken and the residual chlorpyrifos concentration will be determined by high performance liquid chromatography (HPLC).

VI. Response Surface modification for maximum chlorpyrifos degradation

The success of microbial degradation depends on a number of factors, like pH, organic matter, moisture, temperature and nutrient status. Based on the single-factor experiments and Box-Benhnken design, the effect of four factors on the degradation of chlorpyrifos by the obtained microbial strains will be observed. The four factors, including temperature (˚C), oscillator speed (rpm), inoculum concentration (%) and

pH, and their interactions on the degradation of chlorpyrifos will be studied through the use of response surface analysis (Hu *et al*., 2018).

VII. Characterization of chlorpyrifos degrading micro-organisms

- Morphologically distinguishable bacterial and fungal colonies will be observed on Nutrient agar and Czapek Dox Agar plates enriched with chlorpyrifos pesticide.
- These chlorpyrifos degrading isolates will be subjected to morphological, molecular and biochemical studies.

a) Morphological Characteristics of chlorpyrifos degrading strains by processes given in *Bergey's Manual of Systematic Bacteriology*.

S. No.	MORHOLOGICAL CHARACTERISTICS		
	BACTERIA	**FUNGUS**	
		Macromorphological	Micromorphological
1	Gram's Reaction	Texture, Obverse and reverse colour of colony	Dimension of vesicles, phialides, conidia
2	Cell shape	Colony diameter	Shape of vesicles, phialides, conidia
3	Spores	Mycelia, colour of conidia	Texture of vesicles, phialides, conidia
4	Motility	Degree of growth/ Acid – Base production on CREA	Number of branching points between stipe and phialides

b) Molecular identification of chlorpyrifos degrading strains

- 16s rRNA gene sequencing for bacterial strains

  Isolated bacterial strains will be selected and characterized by partial sequencing of the 16sr RNA gene (Rani *et al*, 2007). The genomic DNA will be isolated from bacterial cell using standard CTAB method. The 16s rRNA gene will be amplified by PCR using universal primers 5'-AGAGTRTGATCMTYGTCWAC-3' and 5'-CYGTAMCTTWTTAGCRCT-3' and the purified PCR

product will be outsourced for sequencing of both strands, as described by Das *et al.* (2012).

- 18s r RNA gene sequencing for fungal strains

  The fungi will be identified using molecular approaches of internal transcribed spacer and β-tubulin gene sequencing. Molecular identification of the isolates will be performed using the internal transcribed spacer (ITS) region and partial β-tubulin genes. PCR amplification will be conducted using the ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers for the ITS gene and the Bt2a (5'-GGTAACCAAATCGGTGC TGCTTTC-3) and Bt2b (5'-ACCCTCAGTGTAGTGACCCTTGGC-3') primers for the β-tubulin gene Kim et al., 2012.

c) Biochemical Characteristics of chlorpyrifos degrading strains

These biochemical tests are performed by the process given in *Microbiology: A Laboratory manual by Cappuccino and Sherman*.

S. No.	BIOCHEMICAL CHARACTERISTICS FOR BACTERIA
1	Methyl Red
2	Voges Praskaur's
3	Citrate utilization
4	Catalase
5	Oxidase
6	Casein hydrolysis
7	Starch hydrolysis
8	Gelatin liquefication
9	Indole
10	Lactose/sucrose/glucose utilization
11	De-nitrification/Ammonia production
12	Urease

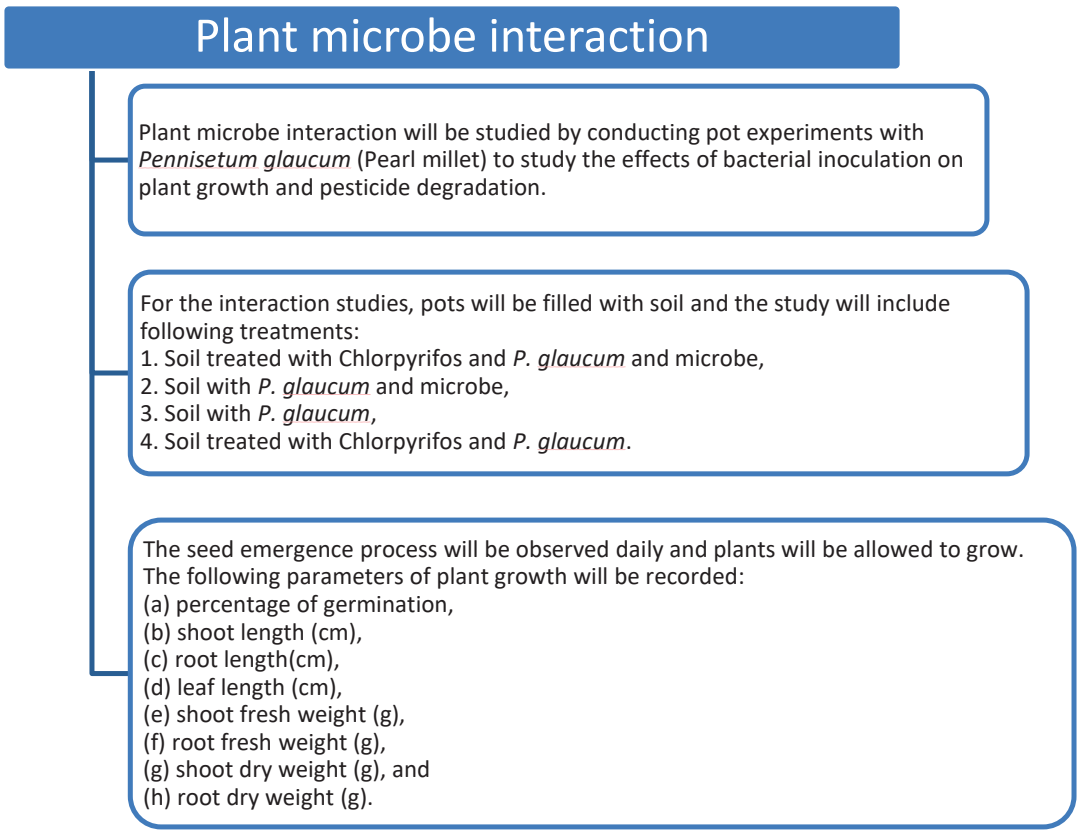
VIII. Study of growth kinetics of chlorpyrifos degrading bacteria

The cultures of chlorpyrifos degrading bacteria will be inoculated in a flask having 100 ml media. These flasks will be incubated at 37°C in a shaking incubator and O.D. at 540nm will be recorded periodically.

Again, the same process will be repeated to understand the growth kinetics of bacteria in the presence of pesticides. The flask with 100ml of media will then be supplemented with chlorpyrifos and the inoculated bacteria will be incubated.

IX. Plant microbe interaction

When the isolated bacteria is inoculated in soil treated with pesticide, we can test whether the bacteria is capable of not only degrading the pesticide in soil but also affects the growth of crop plant growing in that soil. Plant microbe interaction will be studied by conducting pot experiments with *Pennisetum glaucum* to study the effects of bacterial inoculation on plant growth and pesticide degradation. The seeds of the plant will be surface sterilized by treating with 0.1% $HgCl_2$ solution for 5 minutes followed by washing with sterilized distilled water. Chlorpyrifos with a concentration of 200 mg kg^{-1} will be added to soil samples which further will be inoculated with isolated microorganisms. Surface sterilized seeds will be sown in soil samples and proper care of moisture, light and temperature will be taken until seed emergence process is observed.


Plant microbe interaction
Plant microbe interaction will be studied by conducting pot experiments with Pennisetum glaucum (Pearl millet) to study the effects of bacterial inoculation on plant growth and pesticide degradation.
For the interaction studies, pots will be filled with soil and the study will include following treatments:
1. Soil treated with Chlorpyrifos and P. glaucum and microbe,
2. Soil with P. glaucum and microbe,
3. Soil with P. glaucum,
4. Soil treated with Chlorpyrifos and P. glaucum.
The seed emergence process will be observed daily and plants will be allowed to grow. The following parameters of plant growth will be recorded:
(a) percentage of germination,
(b) shoot length (cm),
(c) root length(cm),
(d) leaf length (cm),
(e) shoot fresh weight (g),
(f) root fresh weight (g),
(g) shoot dry weight (g), and
(h) root dry weight (g).

X. Analysis of PGPR activity:

a. Isolation and screening of Phosphate-solubilizing bacteria

To isolate phosphate solubilizing bacteria from soil samples collected, a serial dilution assay will be carried out in 0.9% NaCl solution. 10µL of diluted suspension will be inoculated on Pikovaskya agar medium by spread plate technique and the agar plate will be incubated at 28°C for 7 days.

The isolates showing clear halo around bacterial colonies after incubation period will be considered as P-solubilizing bacteria. Further these bacterial isolates will also be tested for their phosphate solubilizing activity in presence of chlorpyrifos by adding pesticides in Pikovaskya medium and then incubating the inoculated agar plates at 28°C for 7 days. Again, the bacterial colonies showing clear halo around them will be selected.

The extent of P-solubilization will be determined in Pikovaskya broth by chlorostannous-reduced molybdophosphoric acid blue method (King, 1932; Jackson, 1967).

b. Indole acetic acid production

The Indole acetic acid production will be quantitatively analyzed by method of Gordon and Weber (1951). The bacterial strains will be grown on LB medium having fixed concentration of tryptophan. Plates having different pesticide concentrations will be prepared and 0.1ml of culture will be inoculated. The plates will be kept for incubation for 7 days at 28°C with shaking at 125rpm.

After 7 days, 5 ml culture will be centrifuged at 9000g for 15 minutes and an aliquot of 2ml supernatant will be mixed with 100µL of orthophosphoric acid and 4 mL of Salkowsky reagent and incubated at 28°C in darkness for 1 hour. The absorbance will be read at 530nm and IAA concentration will be determined by using calibration curve of pure IAA as standard.

c. Siderophore production

The siderophore production by bacterial colonies will be analyzed by supplementing Chrome azurol S (CAS) agar plates with pesticide. The plates will be spot inoculated with 10µL of 10^8 cells mL^{-1} and will be kept for incubation at 28°C for 4 days. Development of yellow to orange halo around bacterial colonies will indicate siderophore production

SIGNIFICANCE

The increase in demand for agro-products and changing regional climate has escalated consumption and application of pesticides.in current scenario; farmers are using higher doses of pesticides and chemical fertilizers in an indiscriminate manner for higher production of grain yield. This unmanaged usage of such toxic chemicals results in accumulation of their residues in agricultural soils and waters. Such pesticide load causes negative consequences on the microbial flora and fauna which further causes the cascading effects on the agricultural ecosystem structure and function. Because of their unusually long half-life and non-biodegradable nature, these poisonous chemicals are likely to persist in the environment and enter into the food chain and cause serious health hazards.

Pesticide degradation is carried out by several processes described as biotransformation, bio-mineralization, bioaccumulation, biodegradation, bioremediation and co-metabolism. Thus, the microbes which play substantial role in the in situ remediation and detoxification of these toxicants from the concerned environment are of special significance. Effective and indigenous microbial consortia contribute significantly for the removal of toxic pesticides.

Green revolution has marked its importance in India with increased crop production. With increased demand for crops, the consumption of pesticides to kill pests has increased several folds. The widespread use of pesticides is a necessity as a farmer has to bear severe loss because of the destruction caused by pests. The use of pesticides has resulted in problems, caused by their interaction with the biological systems in the environment as they harm soil microorganisms as well as get accumulated in the soil.

Considering the toxic effects of pesticides, it is essential to remove them from the environment. Several methods of pesticide degradation have been employed to remove them from the environment, including chemical methods, but their use made the situation even worse. It is of much importance to make use of biological methods to combat the situation which gives rise to bioremediation. It is the process which makes use of microorganisms which uses xenobiotic compounds like pesticides for their growth, mineralize and detoxify them.

Chlorpyrifos is an organophosphate pesticide, acutely toxic to humans and harms the soil micro flora when get accumulated. It influences the soil microbial activities when applied to it. A modular approach to degrade these pesticides is very essential and use of

microorganisms can be one such approach. Factors like temperature, pH, and available sources of Nitrogen and phosphorus influence the microbes to co-metabolize the pesticide. Therefore, we can say that bioremediation is nature's way of recycling waste and then their reuse by the organisms.

Various bacterial and fungal species have been reported to be able to grow on chlorpyrifos such as *Serratia* sp. and *Pseudomonas* sp. (Cycon *et al.,* 2009), *Paracoccus* sp. and *Enterobacter* strain B-14 (Rani *et al.,* 2008; Xu *et al.,* 2008; Singh *et al.,* 2004; Li *et al.,* 2007; Bhagobaty and Malik, 2008). These bacteria use chlorpyrifos as their sole carbon and energy source thereby reducing their concentration in soil. Bacteria play an important role in degradation due to hydrolytic and oxidative enzymes. It is a viable option for cleaning the pesticide contaminated site as it is the most eco-friendly, highly efficient and cost effective method.

The intermediate metabolite of chlorpyrifos, 3, 5, 6-trichloro-2-pyridinol (TCP) also requires complete degradation as it is toxic. *Alcaligenes faecalis* DSP3, *Sphingobacterium* sp. JAS3, *Ganoderma* sp. JAS4, *Gordonia* sp. JAAS1 were a few microbes that mineralize and degrade the chlorpyrifos and its intermediate TCP (Yang *et al.,* 2005; Silambarasan *et al.,* 2013; Abraham *et al.,* 2013).

Besides pesticides another problem that farmers face is continuous use of fertilizers to meet the increasing demand of crops. Fertilizers increase the yield of crops by providing the required nutrients in optimum amounts to the crop plants. Certainly these fertilizers are the chemical substances which accumulate in the soil and are harmful for environment as they have high biological persistence.

Plant growth promoting activity has been shown by certain chlorpyrifos degrading bacteria and has been reported by various researchers. Bacteria like *Pseudomonas aeruginosa* DKC2, *Acinetobacter calcoaceticus* and yFp tagged *Mesorhizobium sp.* HN3 have shown potent pesticide degrading capability along with plant growth promoting activities.

If the bacteria that degrade chlorpyrifos can also secrete certain metabolites that can promote the plant growth then these bacteria can also act as bio-fertilizers which can be a boon for

farmers. This will reduce their dependency on chemical fertilizers without compromising with the high yields of crop plants.

REFERENCES

1. Agnihotri, V.P., Sinha, A.P. and Singh, K. (1981). Influence of insecticides on soil microorganisms and their biochemical activity. Pesticides, 24: 16-23.
2. Alexander, M. (1981). Non-biodegradable and other recalcitrant molecules. Biotechnology and Bioengineering, 15: 611-647.
3. Anwar, S., Liaquat, F., Khan, Q.M., Khalid, Z.M., and Iqbal, S. (2009) Biodegradation of chlorpyrifos and its hydrolysis product 3, 5, 6-tricholoro-2-pyridinol by Bacillus pumilus strain C2A1. J Hazard Mater 168: 400–405.
4. Bhatia, D., Malik, D.K. (2013). Isolation and characterization of chlorpyrifos degrading soil bacteria of environmental and agronomic significance. *J. Environ. Sci. Eng.,* 55(2):227-238.
5. Bhagobaty, R.K., Joshi, S.R. and Malik, A. (2007). Microbial Degradation of Organophosphorous Pesticide: Chlorpyrifos (Mini-Review). The Internet Journal of Microbiology. Volume 4 Number 1.
6. Briceno, G, Fuentes, M.S., Palma, G. (2012). Chlorpyrifos biodegradation and 3, 5, 6-trichloro-2-pyridinol production by actinobacteria isolated from soil. Int Biodeter Biodegr, 73, 1–7.
7. Carranza, C.S., Aluffi, M.E., Barberis, C.L., Magnoli, C.E. (20016). Evaluation of chlorpyrifos tolerance and degradation by non-toxigenic Aspergillus section flavi strain isolated from agricultural soils. Int.J.Curr.Microbiol.App.Sci (2016) *5*(7): 1-18.
8. CCM press release, China, 2011
9. Chambers, J.E. and Carr, R.L. (1995). Biochemical mechanisms contributing to species differences in insecticidal toxicity. Toxicology*,* 105:291-304.
10. Das, S. and Adhya, T.K. (2015). Degradation of chlorpyrifos in tropical rice soils. Journal of Environmental Management.
11. Devi, P.I., Thomas, J. and Raju, R.K. (2017). Pesticide consumption in India: A spatiotemporal Analysis. Agricultural Economics Research Review Vol. 30 (No.1) January-June 2017 pp 163-172.
12. Times of India, Economic Survey 2017-18.
13. El-Merhibi A., Kumar, A., and Smeaton, T. (2004). Role of piperonyl butoxide in the toxicity of chlorpyrifos to *Ceriodaphnia dubia* and *Xenopus laevis*. Ecotoxicol. Environ. Saf. 57:202-212.

14. Fang, H., Yu, Y., Chu, X., Wang, X., Yang, X., Yu, J., 2009. Degradation of chlorpyrifos in laboratory soil and its impact on soil microbial functional diversity. J. Environ. Sci. 21, 380–386.

15. Fulekar, M.H. and Geetha, M. (2008). Bioremediation of Chlorpyrifos by *Pseudomonas aeruginosa* using scale up technique. *J. of* Appli. *Biosciences,* 12: 657 - 660.

16. Fukuto, T.R., 1990. Mechanism of action of organophosphorus and carbamate pesticides. Environ. Health Perspect. 87, 245-254.

17. Gallo, M. A. and Lawryk, N. J. (1991). Organic phosphorus pesticides: In Handbook of Pesticide Toxicology. Hayes, W. J., Jr. and Laws, E. R., Jr., Eds. Academic Press, New York, NY.

18. Giesy, J.P, Solomon, K.R., Coates, J.R. Doxon, K. R., Giddings, J.M. and Kenaga, E.E. (1999). Chlorpyrifos: Ecological risk assessment in North American environments. Rev. Environ. Contam. Toxicol. 160:1-130.

19. George, A., 2005, Chlorpyrifos degrading bacteria from soils; isolation, screening and selection.

20. Gilani, R.A., Rafique, M., Rehman, A., Munis, M.F.H., Rehman, S., Chaudhary, H.J. (2015). Biodegradation of chlorpyrifos by bacterial genus *Pseudomonas*. J Basic Microbiol 56:105–119

21. Goodman, L.R., Hansen, D.J., Middaugh, D.P., Cripe, G.M. and Moore, J.C. (1985). Method for early life-stage toxicity tests using three atherinid fishes and results with chlorpyrifos.

22. Gong, T., Liu, R., Zuo, Z., Che, Y., Yu, H., Song, C., and Yang, C. (2016) Metabolic engineering of Pseudomonas putida KT2440 for complete mineralization of methyl parathion and c-hexachlorocyclohexane. ACS Synth Biol 5: 434–442.

23. Hamsavathani, V., Aysha, O.S. and Ajith, R. (2017). Isolation and identification of chlorpyrifos degrading bacteria from agricultural soil. Int. J. Adv. Res. 5(5), 1209-122.

24. Hindumathy, C.K., Gayathri, V. 2013. Effect of pesticide (chlorpyrifos) on soil microbial flora and pesticide degradation by strains isolated from contaminated soil. Bioremediation and Biodegradation, 4: 178.

25. Huang, X.D., El-alawi, Y., Pernrose, D.M., Glick,B.R., and Greenberg, B.M., 2004. A multi process phytoremediation system for removal of polycyclic aromatic hydrocarbons from contaminated soils. Environ. Pollut. 130: 465-476.

26. , Ishag, A.E.S.A., Abdelbagi, A.O., Hammad, A.M.A., Elsheikh, E.A.E, Elsaid, O.E., Hur, J.H. and Laing, M.D. (2016). Biodegradation of chlorpyrifos, malathion and dimethoate by three strains of bacteria isolated from pesticide polluted soils in Sudan. J. Agric. Food Chem. 2016, 64, 8491−8498.

27. Jabeen, H., Iqbal, S., Ahmad, F., Afzal, M. and Firdous, S. (2015). Enhanced remediation of chlorpyrifos by ryegrass (*Lolium multiflorum*) and a chlorpyrifos degrading bacterial endophyte *Mezorhizobium* sp. HN3. Int. J. of Phytorem. 6:0.

28. Javaid, M.K., Ashiq, M., and Tahir, M. (2016). Potential of biological agents in decontamination of agricultural soils. Scientifica Vol 2016.

29. Jisha, M.S., John ,E.J., Varghese, E.M. and Krishnasree, N. (2018). In situ Bioremediation of chlorpyrifos by *Klebsiella* sp. isolated from pesticide contaminated agricultural soil. Int.J.Curr.Microbiol.App.Sci *7*(3): 1418-1429.

30. Kahlon, R.S. and Saurabh, G. (2003). To study the effect of bioaugmentation on pesticides contaminated soil by fluorescent *Pseudomonas*, 44th AMI Conference, November 12-14, Dharwad, p.125.

31. Kanekar, P.P., Bhadbhade, B., Deshpande, N.M. and Sarnaik, S.S., 2004, Biodegradation of organophosphorus pesticides. Proceedings of Indian National Science Academy, 70: 57-70.

32. Khanna, S., Vidyalakshmi, C. and Kumar, M. (2009). Biodegradation of Chlorpyrifos in Soil by Enriched Cultures, Curr. Microbiol. 58(1): 35–38.

33. Kumar, R.G., Gopal, A.V., Reddy, R.S. and Chari, D. (2017). Isolation and screening for efficiency of organic phosphorus pesticide (chlorpyrifos) degrading bacteria from different crops. Nat. Env. and Poll. Tech. Vol 16 (169-173).

34. Li, X., He, J., and Li, S. (2007) Isolation of chlorpyrifos degrading bacterium Sphingomonas sp strain Dsp-2 and cloning of the *mpd* gene. Res Microbiol 158: 143–149.

35. Liu, Y.Y., Xiong, Y. (2001). Purification and characterization of a dimethoate-degrading enzyme of *Aspergillus niger* ZHY256 isolated from sewage. Appl. Environ. Microbiol. 67: 3746-3749.

36. Mallick, B. K., Banerji, A., Shakil, N. A. and Sethunathan, N. N. (1999). Bacterial degradation of chlorpyrifos in pure culture and in soil. Bull. Environ. Contam. Toxicol. 62**:**48-55.

37. Raju, N.S. and Supreeth, M. (2017). Biotransformation of chlorpyrifos and endosulfan by bacteria and fungi. Appl Microbiol Biotechnol (2017) 101:5961–5971.

38. Read, D.C., (1976). Comparison of residual toxicities of twenty four registered or candidate pesticides applied to field microplots of soil by different methods. J. Eco. Entomol. 69: 429-437.

39. Romeh, A.A., Hendawi, M.Y. (2014). Bioremediation of certain organophosphorous pesticides by two biofertilizers *Paenibacillus polymyxa and Azospirillum lipoferum.* J Agr. Sci. Tech., 16: 265-276.

40. Sabdono, A. (2007). Biodegradation of chlorpyrifos by a marine bacterium *Bacillus firmus* strain by six associated with branching coral *acropora sp. Coast. Dev.,* 10.

41. Sharma, B., Saxena, S., Datta, A. and Arora, S. (2016). Spectrophotometric analysis of degradation of chlorpyrifos pesticide by indigenous microorganisms isolated from affected soils. Int.J.Curr.Microbiol.App.Sci *5*(9): 742-749.

42. Singh and Abhilash, 2009. Pesticide use and application: An Indian scenario *Journal of Hazardous Materials*, Volume 165, Issue 1, Pages 1-12 P.C. Abhilash, Nandita Singh.

43. Singh, B.K., Walker, A., Morgan, J.A., Wright, D.J. (2004) Biodegradation of chlorpyrifos by *Enterobacter* strain B-14 and its use in bioremediation of contaminated soils. Appl Environ Microbiol 70: 4855–4863.

44. Singh, B.K., Walker, A. (2006). Microbiol degradation of organophosphorous compounds. FEMS Microbiol Rev 30:428–471.

45. Supreeth, M., Chandrashekhar, M.A., Sachin, N., and Raju, N.S. (2016). Effect of chlorpyrifos on soil microbial diversity and its biotransformation by Streptomyces sp. HP-11. Biotech (2016) 6:147.

46. Tashiro, H. and Kuhr, R.J., 1978. Some factors influencing the toxicity of soil applications of chlorpyriphos and diazinon to European chafer grubs. J. Eco. Entomol. 71: 903-907.

47. Thakur, I.S., Kumari, M. and Ghosh, P. (2016). Application of microbes in remediation of hazardous wastes: A review.

48. U.S. Environmental Protection Agency. Pesticide Fact Sheet Number 207: Coumaphos. Office of Pesticides and Toxic Substances, Washington, DC, 1989.5-19

49. U.S. Public Health Service. Hazardous Substance Data Bank. Washington, DC, 1995.5-9

50. Veeraiah, K., Tilak, K.S. (2001). Toxicity and effects of chlorpyrifos to the freshwater fish, *Labeo rohita* (Hamilton). Poll. Res., 20(3): 443-445.

51. Yadav, M., Srivastva, N., Shukla, A.K., Singh, R.S., Upadhyay, S.N., Dubey, S.K. 2015. Efficacy of *Aspergillus* sp. for degradation of chlorpyrifos in batch and continuous aerated packed bed bioreactors. Appl. Biochem. Biotechnol. 175: 16-24.

52. Yang, C., Liu, N., Guo, X., and Qiao, C. (2006) Cloning of *mpd* gene from a chlorpyrifos-degrading bacterium and use of this strain in bioremediation of contaminated soil. FEMS Microbiol Lett 265: 118–125.

53. Yang, L., Zhao, Y., Zhang, B. (2005). Isolation and characterization of a chlorpyrifos and 3, 5, 6-trichloro-2-pyridinol degrading bacterium. FEMS Microbiol Lett, 251, 67–73.

54. Yucel, U., Ylim, M., Gozk, K., Helling, C.S. and Sarykaya, Y. (1999). Chlorpyrifos degradation in Turkish soil. J. Environ. Sci. Heal., 34: 75-95.

55. Zhao, L., Wang, F. and Zhao, J. (2014). Identification and functional characteristics of chlorpyrifos-degrading and plant growth promoting bacterium *Acinetobacter calcoaceticus*. J. of Bas. Microbiol. 54(5):457–463.

56. Zhiyuan, L., Xin, C., Yi, S., and Zhencheng, S.U. (2012). Bacterial Degradation of Chlorpyrifos by *Bacillus cereus*. Advanced Materials Research, 356- 360: 676-680.